Antioxidant and genoprotective activity of selected *cucurbitaceae* seed extracts and LC-ESIMS/MS identification of phenolic components

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ABSTRACT

Cucurbitaceae are one of most widely used plant species for human food but lesser known members have not been examined for bioactive components. The purpose of this study was to evaluate the antioxidant and genoprotective activities from three cucurbitaceae seeds extracts and to identify phenolic components by LC-ESIMS/MS analysis. From the results, the yield of seeds extract was 20-41% (w/w) and samples had 16-40% total phenols as gallic acid equivalents (GAE). Compared with methanol solvent, using acidified methanol led to increased extraction yield by 1.4 to 10-fold, higher phenolic content (149.5±1.2 to 396.4±1.9 mg GAE/g), higher DPPH radical quenching and enhanced genoprotective activity using the pBR322 plasmid assay. LC-ESI-MS/MS analysis led to identification of 14-17 components, based on authentic standards and comparison with literature reports, as mainly phenolic acids and esters, flavonol glycosides. This may be the first mass spectrometric profiling of polyphenol components from cucurbitaceae seeds. **(140words)**

Keywords: *Cucurbitaceae* seeds, total phenols, phenolic glycosides, genoprotective; antioxidant; pBR322 plasmid; LC-ESI-MS/MS analysis.

Highlights:

Cucurbitaceae seeds are novel sources of flavonol glycosides

Cucurbitaceae seeds are sources of phenolic acid and esters

Acidification improves aqueous methanol recovery of cucurbitaceae glycosides

Extracts from Cucurbitaceae seeds possess antioxidant and genoprotective activity

Chemical compounds studied in this article

4-O-Feruloylquinic acid (PubChem CID: 6171347)

Caftaric acid (PubChem CID: 6440397)

Chicoric acid (PubChem CID: 5281764)

Isoquercetin, quercetin 3-O-glucoside (PubChem CID: 5280804)

Kaemferol (PubChem CID: 5280863)

Myricetin (PubChem CID: 5281672)

Quercitrin (Quercetin 3-O-rhamnoside), PubChem CID: 535943

Rosmarinic acid (PubChem CID: 5281792)

Synapic acid (PubChem CID: 10743)

Syringic acid (PubChem CID: 10742)

1. Introduction

The Cucurbitaceae family contains one of most the widely used plant species for human food. The family comprises of 120 genera and 825 species of which 17 genera and 32 species are found in Pakistan. Leaves, fruits and dried seeds from *Cucurbitaceae* (pumpkin, cucumber, melon, watermelon, squash and gourds) are widely consumed. Plants belonging to the *Cucurbitaceae* family may possess pharmacological properties (Talukdar & Hossain, 2014; Vijayakumar, Eswaran, Ojha, Rao Ch & Rawat, 2011), including antidiabetic role (Chandrasekar, Mukherjee & Mukherjee, 1989; Huseini, Darvishzadeh, Heshmat, Jafariazar, Raza & Larijani, 2009; Rashidi, Mirhashemi, Taghizadeh & Sarkhail, 2013), anti-ulcer role, analgesic, nephro-protection (Jain & Singhai, 2010), and anticancer effects (Vijayakumar et al., 2011).Pumpkin (*Cucurbita pepo* L) was extensively investigated for uses in herbal therapy (Abdel-Rahman, 2006) and as a source of bioactive food compounds (Veronezi & Jorge, 2012). Less well-investigated cucurbitaceae are now receiving attention related to both food and medicinal applications (Milind & Kulwant, 2011; Talukdar & Hossain, 2014).

Phytosterols were identified in the *Cucurbitaceae* as biologically active components with antiviral activity (Akihisa, Ghosh, Thakur, Rosenstein & Matsumoto, 1986; Akihisa, Inada, Ghosh,Thakur, Rosenstein, Tamura et al, 1988; Akihisa, Kimura, Kasahara, Kumaki, Thakur & Tamura, 1997; Akihisa, Ogihara, Kato, Yasukawa, Ukiya, Yamanouchi et al, 2001). Antioxidant capacity of Cucurbitaceae seeds was correlated with total phenols (Achu, Fokou, Kansci & Fotso, 2013; Ismail, Chan, Mariod & Ismail, 2010; Talukdar & Hossain, 2014). Koike, Li, Liu, Hata, and Nikaido (2005) and also

Li, Xu, Dou, Chi, Kang and Kuang (2009) identified 5-8 novel phenolic glycoside derivatives of 4-hyroxy benzyl alcohol from different varieties of *Cucurbitaceae* seeds.

There is growing interest in the characterization of plant polyphenols (de Rijke, Out, Niessen, Ariese, Gooijer & Brinkman, 2006; Naczk & Shahidi, 2004; Naczk & Shahidi, 2006; Pérez-Jiménez, Neveu, Vos & Scalbert, 2010). However, phenolic compounds from Cucurbitaceae seeds have not been thoroughly investigated. We previously applied solid phase extraction (SPE) for the isolation of flavonols from plants of medicinal and food value (Sultana & Anwar, 2008; Sultana, Anwar & Przybylski, 2007). The purposes of the study reported in this paper were to, evaluate total phenols content, free radical quenching activity, and genoprotective activities from cucurbitaceae seed extracts. The polyphenol enriched extracts were subjected SPE and LC-ESIMS/MS analysis. This is the first application of LC-MS analysis of cucurbitaceae seed phenols.

2. Materials and methods

2.1. Chemicals and Reagents

Samples of 2,2-diphenyl-1-picryl-hydrazl (DPPH) were obtained from Aldrich Chemical Co. (Steinheim, Germany). Folin–Ciocalteau reagent and gallic acid were purchased from Sigma Chemical Co. (St. Louis, USA). Butylated hydroxytoluene (BHT) and ascorbic acid were procured from Merck (Darmstadt, Germany) while dimethylsulfoxide (DMSO) was from AppliChem (Darmstadt, Germany). pBR322 DNA plasmid was purchased from Fermentas. All chemicals were of HPLC or LC/MS grades.

2.2. Extraction of cucurbitaceae seed phenols

Fruits of Momordica *dioica (*Spinney gourd, Jungli karela*), Citrullus colocynthus* L. (bitter cucumber, desert gourd, egusi,) and *Cucumis melo var. agrestis (mouse melon, chibber, ucado melon,*) were collected from local farms in Faisalabad, Pakistan. The subject species were selected based on their multiple medicinal benefits and their availability in Pakistan. The plants were identified from the Department of Botany, University of Agriculture, Faisalabad, Pakistan. Current nomenclature is available from the USDA germ plasm database (USDA-ARS National Genetic Resources Program, 2015). Seeds were manually separated, washed, shade dried and ground to fine powders. The ground seeds were extracted in an orbital shaker (PA 250/25-H) by sample to solvent ratio of 1:10 (w/v) with methanol/water (70%, 50% and 30% v/v) at room temperature for 24 hours. Seed powders were not defatted prior to antioxidant extraction to avoid losses of nonpolar constituents. Acidified methanol extraction was performed as described previously (Abdel-Aal el & Hucl, 2003; Kim, Kim, Koh, Kim, Lee & Kim, 2008; Sultana et al., 2008; Takeoka, Dao, Full, Wong, Harden, Edwards et al, 1997) with modification. Briefly, samples (10g) of powdered seed were shaken with 100 mL acidified methanol (methanol + 0.5M, 1.0M and 2.0M HCl; 70: 30% v/v) at room temperature for 24 hours. The acidified methanol and non-acidified methanol extracts were concentrated to complete dryness under reduced pressure and stored at 4^{o}C until further analysis.

2.3. Total Phenolic Contents

Non-defatted seed powders were used in this study. Total phenolic contents of all extracts were analyzed using Folin–Ciocalteu reagent. Methanol/water seed extracts (0.5 mL having 1mg dry extract) was mixed with sodium carbonate (2 mL, 7.5%) and Folin–Ciocalteu reagent (2.5 mL, 10%). The mixture was incubated for 30min at room temperature, then absorbance was recorded at 765nm using a UV-VIS spectrophotometer (IRMECO, Geesthacht/Germany, Model 5000). Total phenolic contents were quantified based from absorbance measurements (Abs) and standard curve for gallic acid (2-200 ppm) using the relations below;

$$\text{Total phenols (mg-GAE/ g sample)} = \frac{Abs}{m} * V_{ex} * D_F * \frac{1}{W}$$

where, m (l/mg) = slope from the calibration graph, DF = Dilution factor for sample before assay (1 if undiluted), Vex = Original volume of sample extract and W = dry weight of seed extract (g).

2.4. DPPH Free Radical Scavenging Activity

The antiradical activity of each extract was evaluated following a spectrophotometric DPPH method (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos & Byrne, 2006). Methanolic solutions of each extract (3 mL) at varying concentrations (1-5000µg/mL) were added to methanolic solution of DPPH (1 mL, 0.1mM) and allowed to stand in the dark for 30min at room temperature. Then the absorbance of solution was measured at

517nm (IRMECO 5000) and antioxidant activity was calculated as percentage inhibition of DPPH free radical using the following equation below;

$$\% \text{ reduction (DPPH)} = \left[1 - \frac{Absorbance\ sample}{Absorbance\ of\ DPPH\ soln}\right] * 100$$

the concentration of compound to produce 50% inhibition of DPPH (IC_{50}) for DPPH free radical scavenging were calculated using linear regression analysis; IC5= 50/m.

2.5. PBR322 DNA plasmid protection assay

Genoprotective effects of plant extracts were evaluated qualitatively on supercoiled pBR322 DNA plasmid following a previous method (Tepe, Degerli, Arslan, Malatyali & Sarikurkcu, 2011). In this assay, the protection ability of plant extracts against damage caused by H_2O_2 and UV radiations on DNA plasmid was measured using agarose gel electrophoresis. The irradiation experiments were conducted in Eppendorf tubes containing pBR322 DNA plasmid (3 µL, 172 ng/µL), plant extract (5 µL, in varying concentrations of 5, 10, and 20 mg/mL) and H_2O_2 (2 µL, 30%). A negative control without plant extracts was also run along with sample reactions. All Eppendorf tubes were exposed to UV radiations for 15 min to breakdown the supercoiled DNA plasmid. For electrophoresis analysis, the reaction mixtures were transferred to 0.8% agarose gel along with loading dye (6x) dissolved in Tris Acetate–EDTA buffer (1x). Gels were photographed using gel documentation system (GeneGenius, SYNGENE) after staining with ethidium bromide (0.5µg/mL).

2.6. Purification and LC/MS of optimized seed extracts

The phenolic compounds present in seed extracts optimized as above (Section 2.2-2.5) were investigated by LC/MS technique. First seed extracts were purified and concentrated by solid phase extraction (SPE) and then subjected to LC/MS analysis.

2.6.1. Solid Phase Extraction (SPE)

A multichannel SPE cartridge (ThermoScientific) with Strata C-18 columns and vacuum pump was applied to eliminate non-phenolic compounds. The pre-conditioning of SPE columns was done by 1 mL methanol followed by 1 mL distilled water, in order to remove trapped air and to activate ligands present on sorbent surface. Then, 3.5 mL of plant extracts, diluted in methanol/water (50/50), were loaded onto SPE columns. Washing was performed with 1 mL of distilled water and 1 mL methanol/water (30/70) in order to elute all the impurities without affecting sample analytes. A full vacuum drying was applied for about 5-10 min to remove residual solvent. To recover phenols, cartridges were eluted 1 mL acetonitrile, then 2 mL methanol and finally 2 mL of 5% formic acid in methanol. All three fractions were combined before subjecting to LC/MS (Sun, Liang, Bin, Li & Duan, 2007)

2.6.2. LC-ESI-MS/MS Analysis

Plant extracts purified by SPE were subjected to LC-ESI-MS/MS analysis. This analysis was carried out on liquid chromatography coupled with mass spectrometry (LC/MS) using a ThermoFisher system in which HPLC (Surveyor) system was equipped with linear ESI-Ion Trap (LTQ XL) Mass Spectrometer (ThermoFisher Scientific, San Jose,

CA, USA). Usually, 5µL of sample was injected via an autosampler (Surveyor autosampler plus) in to the HPLC system (Surveyor) equipped with reverse phase C-18 column (Phenomenex 250mm, 5 µm particle size). Sample elution was carried out at flow rate of 5 ml/min using gradient elution comprising Solvent A (water: acetonitrile: trifluoroacetic acid ratio 90:10:0.1% (v/v)) and solvent B (water: acetonitrile: trifluoroacetic acid ratio 10:90:0.06% (v/v). Elution was performed using the following gradient: 0-10 min: 10-35% B, 10-20 min: 35-42 % B and 20-30min: 42-100% B. A photodiode array was used as detector. Prominent peaks were analyzed by mass spectrometer (LTQ XL ThermoFisher Scientific) using atmospheric pressure electrospray ionization (ESI) probe at negative ion mode. Identification of phenols was conducted under full scan mode in the range of 100-600 m/z. MS^2 analysis for each parent ion peak was performed at different Collision Induced Dissociation (CID) powers. X-calibur 1.4 software was applied for calibration of MS data (Sun et al., 2007).

2.7. Statistical analysis

Data are presented as mean ± S.D. of three parallel determinations. Significant difference were analyzed by one way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using MSTAT-C software (version 1.3). Differences among values for were considered statistically significant with $P < 0.05$.

3. Results and discussion

3.1. Extraction yield by mass

Acidified methanol produced a higher yield of seed extract compared to aqueous methanol (Table 1). The mass-yield of extract ranged from 4.0% using aqueous methanol to 72.6% for acidified methanol as solvent. Among three *cucurbitaceae* species, *Momordica dioica* exhibited highest extract yield (28.8±0.2 %) for methanol extraction while *Cucumis melo var. agrestis* showed the highest extract yield (72.6±1.0%) in case of acidified methanol extraction. The extraction yields for various solvents follow the order: 70% methanol > 50% methanol > 30% methanol for non-acidified methanol extraction; and 2.0 M acidified methanol > 1.0 M acidified methanol > 0.5 M acidified methanol for acidified methanol extraction. This different extraction efficiency could be explained by large differences in solubility of various phytochemicals present in these plant species including oils, carbohydrates as well as polyphenols. Many phytochemicals are more soluble in methanol rather than in aqueous medium.

3.2. Total Phenolic Contents

Phenolic acids and polyphenols are important plant secondary metabolites responsible for plant antioxidant activity. Plant phenols can produce antioxidant capacity by a variety of mechanisms, including free radical scavenging, single electron reductions and metal ion chelation (Huang, Ou & Prior, 2005). Previous reports showed that the total phenols content of whole Cucurbita seeds was 0.34-0.4% (w/w) expressed as gallic acid equivalents (Achu et al., 2013; Ismail et al., 2010). In this study (Table 1) the total

phenolic contents for *Cucurbitaceae seeds extracts* ranged from 16% to 40% (w/w) expressed as gallic acid equivalents. For the range of solvents employed in this study, increasing the methanol concentration from 50 to 70 % v/w and acidification of methanol increased the extraction phenolic components. Acidified methanol has been demonstrated to improve the extraction of plant flavonoid glycosides and aglycones, partly due to increasing hydrophobicity of solvent and low pH suppression of polyphenol oxidases (Acosta-Estrada, Gutierrez-Uribe & Serna-Saldivar, 2014; Haghi & Hatami, 2010; Kim et al., 2008; Koh, Youn & Kim, 2014). The mild acidified methanol extraction applied in this investigation is not believed to produce a loss of glycosides (see Section 3.5). Moreover, many polyphenols are ionizable (pKa~7-9) and a low pH solvent would increase the concentration of uncharged species and improve extractability (Wong, Cheung, Lau, Bolanos de la Torre & Owusu-Apenten, 2015).

3.3. DPPH free Radical Scavenging Activity

DPPH is a stable free radical with deep violet color. Radical quenching agents react with DPPH whereby this is reduced to a non-radical yellow colored molecule. In Table 1, acidified methanol plant extracts exhibited significantly ($P \leq 0.05$) higher free radical scavenging activity than non-acidified methanol samples which is consistent with the higher total phenols content (Section 3.2). Previous investigations demonstrated a correlation between total phenols content and DPPH radical quenching activity (Sultana et al., 2007).

3.4. pBR322 DNA plasmid DNA protection assay for oxidative stress

Protective effects of 0.5N acidified methanol and 70% aqueous methanol extracts of *Momordica dioica, Citrullus colocynthus* and *C. melo varagrestis* were evaluated by UV and $^{\bullet}OH$ induced breaks in pBR322 DNA plasmid as *in vitro* assay (Gandhi & Nair, 2005; Sevgi, Tepe & Sarikurkcu, 2015). H_2O_2 in the presence of UV radiations generate hydroxyl radicals that initiate a chain reaction leading to the breakdown of sugar-phosphate backbone of DNA. Hydroxyl radicals also react with nitrogenous bases of nucleic acid, thus, breaking the supercoiled form into linear and open circular form.

Figures 1(a), (b) and (c) represent electropherograms for DNA nicking assay of *Momordica dioica, Citrullus colocynthus* and *C. melo varagrestis,* respectively; the direction of migration is from lower to upper part of the electropherograms. The faster moving band in lane A corresponds to supercoiled circular DNA and slower moving band represents open circular DNA following treatment with H_2O_2 and UV rays. Lanes C-E represent DNA co-treatment with 0.5M acidified methanol extracts of *Momordica dioica* (Fig. 1a)*, Citrullus colocynthus* (Fig. 1b) and *C. melo varagrestis* (Fig. 1c) at concentrations of 5, 10 and 20 mg/mL, respectively. Similarly, lanes F-H show DNA co-treatment by 70% aqueous methanol extracts at concentrations of 5, 10 and 20 mg/mL, respectively. Overall electrophoretic patterns are consistent with the protection supercoiled DNA form in the presence of 0.5 M acidified extract but the DNA protection was less clear following treatment with 70% methanolic extract (lanes F-H).

3.5. LC-ESI-MS/MS Analysis

Tables 2-4 summarize the LC-ESI-MS/MS characterization of 0.5 M acidified methanol extracts. Peaks were identified by reference to retention times, fragmentation patterns and by comparison with published libraries; peaks were also authenticated from molecular weight estimates and supplementary data from the Phenol-Explorer database (Neveu, Pérez-Jiménez, Vos, Crespy, du Chaffaut, Mennen et al., 2010; Pérez-Jiménez et al., 2010).

HPLC analysis of SPE isolates from *Momordica dioca* led to identification of fourteen components with a mass range of 160-600 *amu* (Table 2). Figure 2 shows a sample LC/MS data for *Momordica dioca* extracts; Peak 13 (RT 26.46 min) showing MS peak at m/z 311.07 (Figure 2) indicated the presence of caftaric acid. The parent ion peak was subjected to CID fragmentation to give three daughter ions at m/z 179, 149 and 135. First peak (m/z 179) corresponded to molecular ion of caffeic acid by losing tartaric acid residue, second peak (m/z 149) remained unidentified while a third peak (m/z 135) corresponded to a decarboxylated caffeic acid.

The components isolated from Cucurbita seed extracts could be grouped into two broad classes, (a) phenolic acids and esters and (b) flavonoids and flavonoid glycosides. The phenolic acids mainly hydroxycinnamic acid derivatives were (Table 2); methyl ellagic acid (peak 4), ellagic acid (peak 7), rosmarinic acid (peak 10), caftaric acid (peak 13) and 4-feruloylquinic acid (peak 15). The flavonols were represented by galangin (peak 6), quercetin (peak 8) and myricetin (peak 14). Flavonol glycosides were identified as naringenin 7-O-glucoside (peak 3), apigenin 7-O-glucuronide (peak 11),

myricetin 3-O-glucoside (peak 12), quercitrin (peak 17) and myricitrin or myricetin-3-O-rhamnoside (peak 18). As an example, naringenin-7-O-glucoside (peak 3) with a RT of 4.23 min produced an MS peak at m/z 433.11. This parent ion then fragmented to give two peaks at m/z 271 *amu* (by loss of glucose residue) and other at m/z 153 which is characteristic of naringenin as reported by previously (Pfundstein, El Desouky, Hull, Haubner, Erben & Owen, 2010). The other glycosides were identified similarly according their retention time and MS/MS patterns. Quercetrin (quercetin 3-O-rhamnoside) was the highest concentration (532 ppm) amongst the identified phenolic constituents of *Momordica dioca.* Caftaric acid (caffeic acid ester with tartaric acid; 460.16 ppm); myricetin aglycone (439.78 ppm); myricitrin (myricetin 3-O-rhamnoside; 423.19 ppm) and 4-feruloylquinic acid (423.48 ppm) were also present in considerable higher concentrations.

LC-MS/MS analysis results for C*itrullus colocynthus* (Table 3) showed that components were, (a) phenolic acids or phenolic acid conjugates or (b) flavonol glycosides. The phenolic acids were represented by vanillic acid (peak 1), sinapic acid (peak 4), ferulic acid (peak 5) and ellagic acid (peak 6). Examples of phenolic acid conjugates (peaks 3, 9, and 19) were protocatechuic (peak 3), caffeoyl glucose (peak 9) and chicoric acid (peak 19). Flavonol (peaks 8, 12 and 15) and flavonol glycosides apigenin-7-glucoside (peak 10), myricetin 3-O-glucoside (peak 11), kaempferol-3-rutinoside (peak 13), myrictrin (myricetin 3-O-rhamnoside; peak 14) and isoquercitrin (quercetin 3-O-glucoside; peak 16) were also present. Quantitative analysis showed (Table 3) that dicaffeoyl tartaric acid (chicoric acid; 454.92 ppm), sinapic acid (409.51

ppm) kaempferol (394.08 ppm), isoquercetrin (392.62 ppm) and luteolin (329.74 ppm) were present in quite high concentrations.

For *Cucumis melo var. agrestis* LC/MS analysis of material eluted from SPE separation indicated twelve phenolic compounds comprising, (a) phenolic acids and their conjugates (peaks 2, 6, 3, 4, 8, 10 and 14) and (b) flavonol and their derivatives (peaks 7,9, 13). The former group were represented by syringic acid (peak 2), ferulic acid (peak 6), methyl gallate, (peak 3), sinapic acid hexoside (peak 4), caffeoyl glucose (peak 8), 1,6-digalloyl glucose (peak 10), glycosyringic acid (peak 14). The flavonoids and their derivatives included chrysin (peak 7), bis-methylated quercetin (peak 1), quercitrin (peak 9), isoquercitrin (peak 11), malvidin-3-O-glucoside (peak 13). From the quantitative analysis (Table 4) then glycosyringic acid (445.60 ppm), malvidine-3-O-glucoside (399.61 ppm), quercetrin (344.29 ppm) and bis-methylated quercetin (343.71 ppm) were most abundant compounds of all bioactive compounds found acidified methanol extract of *Cucumis melo varagrestis.* Other phenolic compounds were also present in quite reasonable concentrations (250-300ppm) as demonstrated in Table 4.

Acidified methanol solvent extraction is thought to avoid the enzyme catalyzed oxidation of polyphenols as well as increasing the release of compounds bound by physical forces (Abdel-Aal el et al., 2003; Acosta-Estrada et al., 2014; Kim et al., 2008; Sultana et al., 2008; Sultana et al., 2007). Indeed, the use of mild acidified methanol extraction is a common approach for recovery of anthocyanin glycosides. By contrast, conversion anthocyanin to the aglycone state form required treatment with 6N HCl at 100 ^{o}C for 30 min – 4 hours (Abdel-Aal el et al., 2003; Kim et al., 2008; Takeoka et al., 1997). Interestingly, whilst *Momordica dioica* contained quercitrin (quercetrin-3-O-

rhamnoside) it was quercetrin-3-O-glucoside (isoquercitrin) derivative which was found in *Citrullus colocynthus*.

Some study limitations with relevance for data interpretation are worth noting. Though polyphenolic compounds from Cucurbitaceae seeds were profiled, it is not certain that extracts purified by SPE are representative of all classes of compounds. Tables 2-4 show the predominant components isolated under the present method (acidified methanol extraction and SPE) were flavonol/ flavonol glycosides and phenolic acids and derivatives. Some flavanones (hesperetin) and flavones (chrysin) occurred in lower concentrations. Other classes of polyphenols were not presented in the SPE isolated sample. A further interesting feature is that though flavonol glycosides were present in higher concentrations compared to aglycones, the former did not reach 99-100% glycoside distribution noted in other sources (Pérez-Jiménez et al., 2010). The presence of quercetin glycosides in high quantities is notable as these have enhanced bioavailability compared to the corresponding aglycones (Crespy, Morand, Besson, Manach, Démigné & Rémésy, 2001). The current investigations also do not allow estimates of the different components in unextracted foods of a fresh weight basis.

4. Conclusions

The antioxidant and genoprotective activity for polyphenol extracts of *Momordica dioica, Citrullus colocynthus* and *C. melo var. agrestis* were analyzed. The acidified methanol extraction yielded greater amounts of extracts, a higher polyphenol contents, higher antioxidant activity and increased genoprotective activity. *Citrullus colocynthus*

showed the highest phenolic contents and thus highest antioxidant potential. To our knowledge this is the first investigation of Cucurbit seed polyphenol constituents using LC-MS/MS analysis. The results indicated the presence of phenolic acids, flavonoids and flavonoid glycosides. It is concluded that Cucurbita seeds may be important sources of antioxidant compounds and also a range of phytochemicals with possible nutraceutical uses.

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Conflict of interest

All authors declare no conflict of interest.

References

Abdel-Aal el, S. M., & Hucl, P. (2003). Composition and stability of anthocyanins in blue-grained wheat. *Journal of Agriculture and Food Chemistry*, *51*(8), 2174-2180.

Abdel-Rahman, M. K. (2006). Effect of Pumpkin Seed (Cucurbita pepo L.) Diets on Benign Prostatic Hyperplasia (BPH):Chemical and Morphometric Evaluation in Rats. *World Journal of Chemistry 1 (1):*, *1*(1), 33-40.

Achu , M. B., Fokou, E., Kansci, G., & Fotso, M. (2013). Chemical evaluation of protein quality and phenolic compound levels of some Cucurbitaceae oilseeds from Cameroon. *African Journal of Biotechnology Vol. 12(7), pp. 735-743,*, vol. 2015.

Acosta-Estrada, B. A., Gutierrez-Uribe, J. A., & Serna-Saldivar, S. O. (2014). Bound phenolics in foods, a review. *Food Chemistry*, *152*, 46-55.

Akihisa, T., Ghosh, P., Thakur, S., Rosenstein, F. U., & Matsumoto, T. (1986). Sterol compositions of seeds and mature plants of family cucurbitaceae. *Journal of the American Oil Chemists Society*, *63*(5), 653-658.

Akihisa, T., Inada, Y., Ghosh, P., Thakur, S., Rosenstein, F. U., Tamura, T., & Matsumoto, T. (1988). Compositions of triterpene alcohols of seeds and mature plants of family cucurbitaceae. *Journal of the American Oil Chemists Society*, *65*(4), 607-610.

Akihisa, T., Kimura, Y., Kasahara, Y., Kumaki, K., Thakur, S., & Tamura, T. (1997). 7-oxodihydrokarounidiol-3-benzoate and other triterpenes from the seeds of cucurbitaceae. *Phytochemistry*, *46*(7), 1261-1266.

Akihisa, T., Ogihara, J., Kato, J., Yasukawa, K., Ukiya, M., Yamanouchi, S., & Oishi, K. (2001). Inhibitory effects of triterpenoids and sterols on human immunodeficiency virus-1 reverse transcriptase. *Lipids*, *36*(5), 507-512.

Chandrasekar, B., Mukherjee, B., & Mukherjee, S. K. (1989). Blood sugar lowering potentiality of selected Cucurbitaceae plants of Indian origin. *Indian Journal of Medical Research*, *90*, 300-305.

Crespy, V., Morand, C., Besson, C., Manach, C., Démigné, C., & Rémésy, C. (2001). Comparison of the intestinal absorption of quercetin, phloretin and their glucosides in rats. *The Journal of Nutrition*, *131*(8), 2109-2114.

de Rijke, E., Out, P., Niessen, W. M., Ariese, F., Gooijer, C., & Brinkman, U. A. (2006). Analytical separation and detection methods for flavonoids. *Journal of Chromatography A*, *1112*(1-2), 31-63.

Gandhi, N. M., & Nair, C. K. K. (2005). Protection of DNA and membrane from gamma radiation induced damage by gallic acid. *Molecular and Cellular Biochemistry*, *278*(1-2), 111-117.

Haghi, G., & Hatami, A. (2010). Simultaneous quantification of flavonoids and phenolic acids in plant materials by a newly developed isocratic high-performance liquid chromatography approach. *Journal of Agricultural and Food Chemistry*, *58*(20), 10812-10816.

Huang, D., Ou, B., & Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. *Journal of Agricultural and Food Chemistry*, *53*(6), 1841-1856.

Huseini, H. F., Darvishzadeh, F., Heshmat, R., Jafariazar, Z., Raza, M., & Larijani, B. (2009). The clinical investigation of *Citrullus colocynthis* (L.) schrad fruit in treatment of Type II diabetic patients: a randomized, double blind, placebo-controlled clinical trial. *Phytotherapy Research*, *23*(8), 1186-1189.

Ismail, H. I., Chan, K. W., Mariod, A. A., & Ismail, M. (2010). Phenolic content and antioxidant activity of cantaloupe (*Cucumis melo*) methanolic extracts. *Food Chemistry*, *119*(2), 643-647.

Jain, A., & Singhai, A. K. (2010). Effect of Momordica dioica Roxb on gentamicin model of acute renal failure. *Natural Product Research*, *24*(15), 1379-1389.

Kim, M. K., Kim, H. A., Koh, K., Kim, H. S., Lee, Y. S., & Kim, Y. H. (2008). Identification and quantification of anthocyanin pigments in colored rice. *Nutrition Research and Practice*, *2*(1), 46-49.

Koh, K., Youn, J. E., & Kim, H. S. (2014). Identification of anthocyanins in black soybean (Glycine max (L.) Merr.) varieties. *Journal of Food Science and Technology*, *51*(2), 377-381.

Koike, K., Li, W., Liu, L., Hata, E., & Nikaido, T. (2005). New phenolic glycosides from the seeds of *Cucurbita moschata*. *Chemical and Pharmaceutical Bulletin (Tokyo)*, *53*(2), 225-228.

Li, F. S., Xu, J., Dou, D. Q., Chi, X. F., Kang, T. G., & Kuang, H. X. (2009). Structures of new phenolic glycosides from the seeds of *Cucurbita moschata*. *Natural Products Communication*, *4*(4), 511-512.

Milind, P. and S. Kulwant. (2011). "Musk melon is eat-must melon." Retrieved June, 2015, from www.irjponline.com/admin/php/uploads/vol-2_issue-8/10.pdf.

Naczk, M., & Shahidi, F. (2004). Extraction and analysis of phenolics in food. *Journal of Chromatography A*, *1054*(1-2), 95-111.

Naczk, M., & Shahidi, F. (2006). Phenolics in cereals, fruits and vegetables: occurrence, extraction and analysis. *Journal of Pharmaceutical and Biomedical Analysis*, *41*(5), 1523-1542.

Neveu, V., Pérez-Jiménez, J., Vos, F., Crespy, V., du Chaffaut, L., Mennen, L., Knox, C., Eisner, R., Cruz, J., Wishart, D., Scalbert, A (2010, January 1, 2010). "Phenol-Explorer: an online comprehensive database on polyphenol contents in foods." Database Retrieved July, 2015, from http://database.oxfordjournals.org/content/2010/bap024.abstract.

Pérez-Jiménez, J., Neveu, V., Vos, F., & Scalbert, A. (2010). Systematic analysis of the content of 502 polyphenols in 452 foods and beverages: an application of the phenol-explorer database. *Journal of Agricultural and Food Chemistry*, *58*(8), 4959-4969.

Pfundstein, B., El Desouky, S. K., Hull, W. E., Haubner, R., Erben, G., & Owen, R. W. (2010). Polyphenolic compounds in the fruits of Egyptian medicinal plants (*Terminalia bellerica, Terminalia chebula* and *Terminalia horrida*): characterization, quantitation and determination of antioxidant capacities. *Phytochemistry*, *71*(10), 1132-1148.

Rashidi, A. A., Mirhashemi, S. M., Taghizadeh, M., & Sarkhail, P. (2013). Iranian medicinal plants for diabetes mellitus: a systematic review. *Pakistan Journal of Biological Sciences, 16*(9), 401-411.

Sevgi, K., Tepe, B., & Sarikurkcu, C. (2015). Antioxidant and DNA damage protection potentials of selected phenolic acids. *Food and Chemical Toxicology*, *77*, 12-21.

Sultana, B., & Anwar, F. (2008). Flavonols (kaempeferol, quercetin, myricetin) contents of selected fruits, vegetables and medicinal plants. *Food Chemistry*, *108*(3), 879-884.

Sultana, B., Anwar, F., & Przybylski, R. (2007). Antioxidant activity of phenolic components present in barks of *Azadirachta indica, Terminalia arjuna, Acacia nilotica*, and *Eugenia jambolana* Lam. trees. *Food Chemistry*, *104*(3), 1106-1114.

Sun, J., Liang, F., Bin, Y., Li, P., & Duan, C. (2007). Screening non-colored phenolics in red wines using liquid chromatography/ultraviolet and mass spectrometry/mass spectrometry libraries. *Molecules*, *12*(3), 679-693.

Takeoka, G. R., Dao, L. T., Full, G. H., Wong, R. Y., Harden, L. A., Edwards, R. H., & Berrios, J. D. J. (1997). Characterization of Black Bean (Phaseolus vulgaris L.) Anthocyanins. *Journal of Agricultural and Food Chemistry*, *45*, 3395-3400

Talukdar, S. N., & Hossain, M. N. (2014). Phytochemical, phytotherapeutical and pharmacological study of *Momordica dioica*. *Evidence-Based Complementary and Alternative Medicine 2014 (2014), Article ID 806082, 11 pages*, vol. 2015.

Tepe, B., Degerli, S., Arslan, S., Malatyali, E., & Sarikurkcu, C. (2011). Determination of chemical profile, antioxidant, DNA damage protection and antiamoebic activities of *Teucrium polium* and *Stachys iberica*. *Fitoterapia*, *82*(2), 237-246.

Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., & Byrne, D. H. (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. *Journal of Food Composition and Analysis*, *19*(6-7), 669-675.

USDA-ARS National Genetic Resources Program (2015). Taxon: Cucumis melo L. subsp. agrestis (Naudin) Pangalo var. agrestis Naudin. *Germplasm Resources Information Network - (GRIN) [Online Database].National Germplasm Resources Laboratory, Beltsville, Maryland.*, vol. 2015.

Veronezi, C. M., & Jorge, N. (2012). Bioactive compounds in lipid fractions of pumpkin (Cucurbita sp) seeds for use in food. *Journal of Food Science*, *77*(6), C653-657.

Vijayakumar, M., Eswaran, M. B., Ojha, S. K., Rao Ch, V., & Rawat, A. K. (2011). Antiulcer activity of hydroalchol extract of *Momordica dioica* roxb. Fruit. *Indian Journal of Pharmaceutical Sciences*, *73*(5), 572-577.

Wong, C. W., Cheung, W. S. M., Lau, Y. Y., Bolanos de la Torre, A. A. S., & Owusu-Apenten, R. (2015). A FRAP assay at pH 7 unveils extra antioxidant activity from green, black, white and rooibos tea but not apple tea. *Food and Nutrition Report* 1(1) Retrieved Aug, 2015, from http://verizonaonlinepublishing.com/FnNPDF/FoodandNutritionReport3.pdf.

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Table 1: Extract yields (%age), total phenolic contents (GAE, mg/g) and %age DPPH scavenging activity of non-hydrolyzed and hydrolyzed extracts of *M. dioica, C. colocynthus* L. and *C. melo varagrestis*.

	Momordica dioica			***Citrullus colocynthus* L.**			***Cucumis melo varagrestis***		
solvent	Extract Yield (%)[A]	TPC[B]	%age Scavenging[C]	Extract Yield (%)[A]	TPC[B]	%age Scavenging[C]	Extract Yield (%)[A]	TPC[B]	%age Scavenging[C]
70% Meth	28.8±0.2 [b]	228.6±1.9 [d]	91.62±1.3 [b]	15.8±0.2 [d]	251.4±2.3 [b]	89.21±1.1 [d]	5.4±0.07 [d]	41.6±0.9 [d]	72.76±1.4 [d]
50% Meth	21.5±0.3 [c]	220.2±1.7 [e]	89.56±1.4 [c]	15.3±0.1 [e]	237.2±2.4 [b]	90.21±1.0 [d]	4.5±0.06[de]	34.8±0.4 [e]	76.85±1.6 [c]
30% Meth	20.0±0.1 [d]	208.0±1.8 [f]	84.92±1.1 [d]	15.1±0.2 [e]	226.4±2.0 [c]	93.05±1.2 [c]	4.0±0.04 [e]	31.8±0.6 [f]	79.45±1.0 [b]
2.0M Acid, Meth	40.4±0.6 [a]	337.5±2.4 [c]	91.91±1.0 [b]	27.0±0.3 [a]	384.4±2.5 [a]	96.21±1.0 [b]	72.6±1.0 [a]	149.5±1.2 [c]	81.25±1.2 [b]
1.0M Acid.Meth	40.5±0.5 [a]	346.2±2.0 [b]	94.01±0.9 [a]	22.6±0.2 [b]	391.2±2.1 [a]	96.89±1.0 [b]	67.3±1.1 [b]	158.8±1.0 [b]	85.99±0.5 [a]
0.5M Acid.Meth	40.7±0.5 [a]	354.4±1.9 [a]	94.48±0.9 [a]	21.6±0.3 [c]	396.4±1.9 [a]	98.86±1.1 [a]	57.4±1.0 [c]	164.7±1.1 [a]	86.21±0.9 [a]

Values are mean ± SD of three replications. Solvents are, methanol (Meth) or acidified methanol (Acid.Meth).

Different letters in each column represent significant differences ($p \leq 0.05$) among solvents used.

[A] Yield % (w/w dry biomass).

[B]TPC, total phenolic contents expressed as mg gallic acid equivalents (GAE)/g extract.

[C] Value %age Scavenging by extract concentration 5 mg/mL.

Table 2: LC-ESI-MS/MS characterization of 0.5M acidified methanol extracts from *Momordica dioica (*Spiney gourd, Jungli karela*)*

Peak No.	RT(min)	MW	$[M-H]^-$	MS^2 Ions	Identified Compounds	Molecular Formula**	Conc.* (ppm)	AAuth
5	9.5	_____	161.1	89	Unknown	_____	259.64	
2	3.06	_____	333.32	273	Unknown	_____	261.38	
4	4.83	316.22	315.11	301,257	Methyl ellagic acid	$C_{15}H_8O_8$	261.76	Y
3	4.23	434.4	433.11	271,153	Naringenin-7-O-glucoside	**$C_{21}H_{22}O_{10}$**	263.25	Y
6	21.84	270.24	269.04	227,197	Galangin	$C_{15}H_{10}O_5$	290.44	Y
9	23.79	_____	303.07	285	Unknown	_____	298.01	
8	23.43	302.24	300.98	179,151	Quercetin	$C_{15}H_{10}O_7$	313.73	
1	0.02	302.27	301.07	258,143	Hesperetin	$C_{16}H_{14}O_6$	320.71	Y
7	22.65	302.2	301.03	257	Ellagic acid	$C_{14}H_6O_8$	341.97	Y
10	24.27	360.31	359.12	161,197	Rosmarinic acid	$C_{18}H_{16}O_8$	358.85	Y
12	25.63	480.38	479.05	317,179	Myricetin-3-O-glucoside	**$C_{21}H_{20}O_{13}$**	378.65	Y
16	28.26	_____	293.1	259	Unknown	_____	407.76	
11	24.79	446.34	445.04	269, 175	Apigenin-7-O-glucuronide	**$C_{21}H_{18}O_{11}$**	411.83	Y
18	29.29	464.38	463.12	316	Myricitrin	**$C_{21}H_{20}O_{12}$**	423.19	
15	27.5	368.11	367.06	173, 191	4-Feruloyl-quinic acid	$C_{17}H_{20}O_9$	423.48	Y
14	27.1	318.24	317.08	179, 151	Myricetin	$C_{15}H_{10}O_8$	439.78	
13	26.46	312.23	311.07	149, 179, 135	Caftaric acid	**$C_{13}H_{12}O_9$**	460.16	Y
17	28.92	448.38	447.14	301, 179, 151	Quercitrin	**$C_{21}H_{20}O_{11}$**	532.07	Y
19	29.74	_____	427.05	409	Unknown	_____	573.11	

*Ranked by concentration (ppm), ** glycosides are shown in bold, Y = Peak authenticated by published resources.

Tasble 3. LC-ESI-MS/MS characterization of 0.5M acidified methanol extracts from *Citrullus colocynthus L. (bitter cucumber, desert gourd, egusi,)*

Peak No.	RT(min)	MW	$[M-H]^-$	MS^2 Ions	Identified Compounds	Molecular Formula**	Conc.* (ppm)	Auth
7	6.18	_____	327.25	309	Unknown	_____	254.16	
1	2.31	168.14	167.14	152,123	Vanillic acid	$C_8H_8O_4$	254.86	Y
3	3.39	316.26	315	153, 09	Protocatechuic acid hexoside	$C_{13}H_{16}O_9$	254.89	Y
9	21.56	342.3	340.96	179, 35	Caffeoyl glucose	**$C_{15}H_{18}O_9$**	255.74	Y
10	22.7	432.38	431.07	269	Apigenin-7-glucoside	$C_{21}H_{20}O_{10}$	256.43	Y
2	2.96	176.12	175.01	115	Ascorbic acid	$C_6H_8O_6$	256.52	
8	10.37	270.24	268.97	119, 53, 243	Apigenin	$C_{15}H_{10}O_5$	256.87	Y
11	24.73	480.38	478.96	317, 79, 151	Myricetin 3-O-glucoside	**$C_{21}H_{20}O_{13}$**	259.05	Y
6	4.88	302.2	301.05	257	Ellagic acid	$C_{14}H_6O_8$	263.42	Y
12	25.52	270.28	268.98	197, 33	Alpinetin	$C_{16}H_{14}O_4$	264.59	Y
5	4.6	194.18	193.09	179, 49,134	Ferulic acid	$C_{10}H_{10}O_4$	266.3	
13	26.43	594.52	593.16	285,325	Kaempferol-3-rutinoside	**$C_{27}H_{30}O_{15}$**	279.37	Y
14	27.69	464.38	463.11	316	Myricitrin	$C_{21}H_{20}O_{12}$	311.69	Y
15	27.95	286.24	285.06	213,151, 133	Luteolin	$C_{15}H_{10}O_6$	329.74	Y
16	28.37	464.38	463.16	301	Isoquercitrin	**$C_{21}H_{20}O_{12}$**	392.62	Y
17	28.84	286.24	285.08	241,169,151	Kaempferol	$C_{15}H_{10}O_6$	394.08	
4	4.24	224.21	223.02	208, 179,164	Sinapic acid	$C_{11}H_{12}O_5$	409.51	
18	29.65	_____	334.92	351	Unknown	_____	409.8	
19	29.85	474.37	472.96	311, 179	Chicoric acid	$C_{22}H_{18}O_{12}$	454.92	

*Ranked by concentration (ppm), **phenolic glycosides are shown in bold, Y = Peak authenticated by published resources.

Table 4. LC-ESI-MS/MS characterization of 0.5M acidified methanol extracts from *Cucumis melo var. agrestis (mouse melon, chibber, ucado melon*)

Peak No.	RT(min)	MW	$[M-H]^-$	MS^2 Ions	Identified Compounds	Molecular Formula**	Conc* (ppm)	Auth
7	5.46	254.24	253.19	181,151, 101	Chrysin	$C_{15}H_{10}O_4$	257.48	Y
6	5.06	194.18	193.14	179,149, 134	Ferulic acid	$C_{10}H_{10}O_4$	258.06	Y
10	24.03	484.36	482.98	313,169	1,6-Di-O-galloyl glucoside	$C_{20}H_{20}O_{14}$	258.88	Y
5	4.62	_____	288.06	244	Unknown	_____	259.11	
3	3.36	184.15	183.16	169, 125	Methyl Gallate	$C_8H_8O_5$	259.75	Y
11	24.79	464.38	463.05	301	Isoquercetin	$C_{21}H_{20}O_{12}$	264.59	Y
8	22.26	342.3	340.92	179, 135	Caffeoyl glucose	**$C_{15}H_{18}O_9$**	269.8	Y
4	4.36	386.35	385.04	223	Sinapic acid hexoside	**$C_{17}H_{22}O_{10}$**	289.85	Y
2	0.87	198.17	197.1	182, 153	Syringic acid	$C_9H_{10}O_5$	290.15	
12	25.58	_____	261.14	199	Unknown	_____	334.98	
1	0.02	330.27	329.15	315, 165	Bis-methylated quercetin	$C_{17}H_{14}O_7$	343.71	Y
9	22.9	448.38	447.08	301, 79, 151	Quercitrin	**$C_{21}H_{20}O_{11}$**	344.29	Y
13	26.5	494.14	493.18	331	Malvidin-3-O-glucoside	**$C_{23}H_{26}O_{12}$**	399.61	Y
14	28.28	360.31	359.15	197	Glycosyringic acid	$C_{15}H_{20}O_{10}$	445.6	Y

*Ranked by concentration (ppm), **phenolic glycosides are shown in bold, Y = Peak authenticated by published resources.

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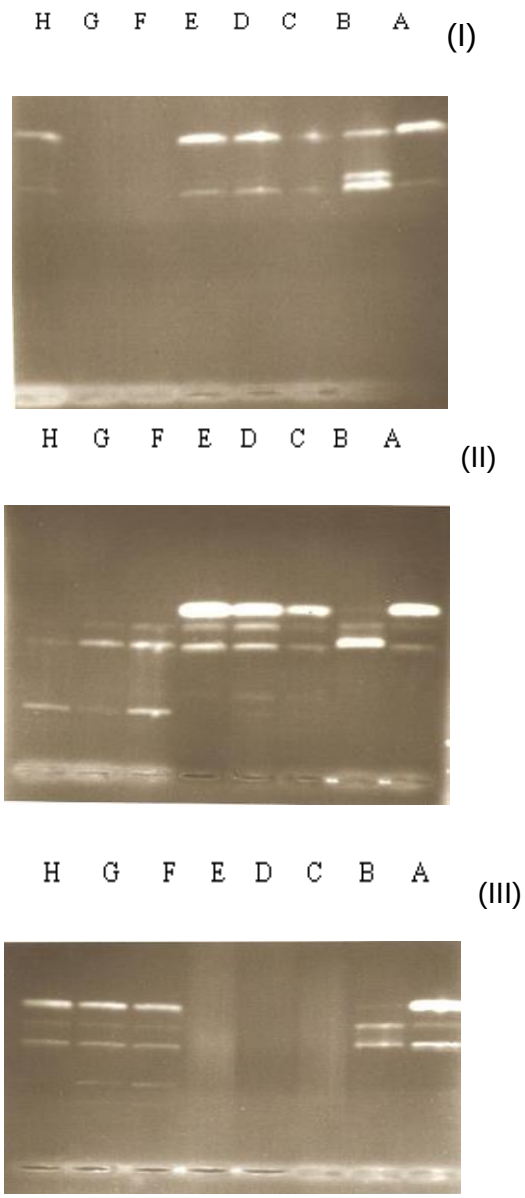

Figure 1: Electropherograms for DNA protection assay for oxidative stress using extracts from (I) *Momordica dioica*, (II) *Citrullus colocynthus L*. and (III) *Cucumis melo var. agrestis*. The direction of migration is upwards. For Figure I-III, lane A = untreated DNA plasmid, lane B = DNA plasmid+ H_2O_2/UV treatment, lane C-E = lane B+ 0.5M acidified methanol extract, Lane F-H = lane B +70% aqueous methanol extract. Treatment concentration was 5, 10 and 20 mg/ml in lanes C-E, and F-H.

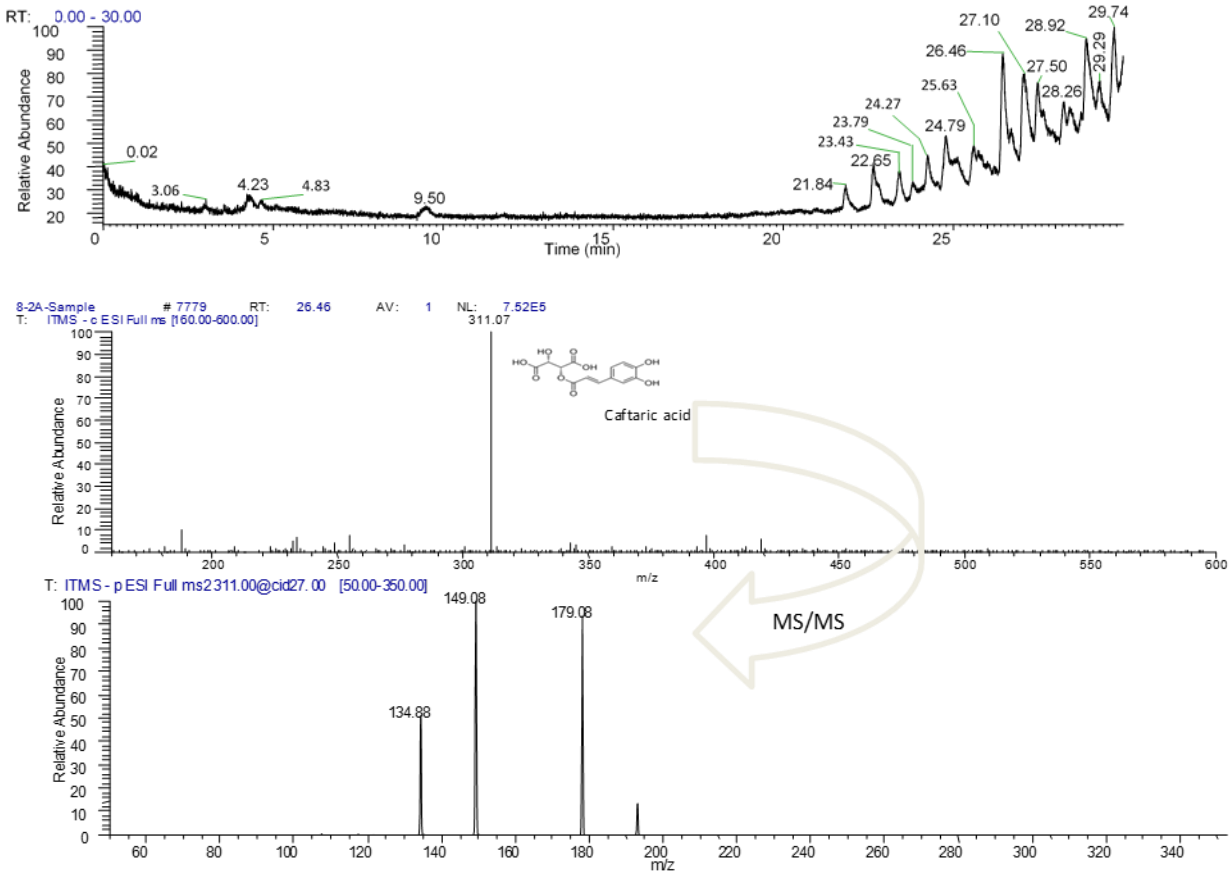

Figure 2: Sample LC/MS/MS data for *Momordica dioca extract.* Peak with a retention time 26.46 min was analyzed with an MS peak at m/z 311.07 indicating the presence of Caftaric acid and confirmed by MS/MS fragments (see text for details).